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Glycine-rich cell wall proteins act as specific antigen targets in autoimmune and food allergic disorders

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Abstract

Our objective was to investigate the presence of a B and T cell immune response directed against the glycine-rich cell wall protein (GRP) in patients with different autoimmune disorders and with food allergy. GRP is an ubiquitous food protein that has high homology with cytokeratins and other self proteins [Epstein–Barr virus nuclear antigen-1 (EBNA-1), heterogeneous nuclear ribonucleoprotein, fibrillar collagen] which are common targets in autoimmune disorders. A peptide (GGYGDGGAHGGGYGG) derived from GRP was used to screen human sera in direct and competitive ELISA assay. Anti-GRP-specific IgG were analyzed for their ability to cross-react with autoantigens. The intracellular cytokine profiles of the peptide-specific T cell clones obtained from representative patients have been studied. BALB/c mice were immunized with the peptide coupled to the carrier protein keyhole limpet hemocyanin (KLH). Serum IgG antibodies directed against the GRP peptide were detected in several autoimmune disorders and in food allergic patients, and were able to cross-react with autoantigens including keratin, collagen and EBNA-1. Twenty-five T cell clones showed a specific proliferative response to the GRP peptide and were of the T_h0 phenotype. Eight of the 10 BALB/c mice immunized with the peptide coupled to KLH developed an autoimmune response. Our data suggest that phylogenetically highly conserved epitopes in plants, viruses and humans may be responsible for an

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autoimmune response in susceptible individuals. They also indicate that the antigen spreading of a particular sequence among apparently divergent proteins may participate to initiate or amplify an immune response.

Keywords: autoantigens, glycine-rich protein, random peptide library

► Introduction

The extensive search for pathogenetically relevant autoantigen targets in autoimmune diseases has been disappointing so far. Different reasons may account for this difficulty: (i) different autoantigenic epitopes may be important for each patient depending on the MHC background, (ii) the set of the encountered foreign antigens may select and activate particular cross-reacting autoreactive T cells (1), (iii) the local inflammation can induce the release of additional self-antigens and/or epitope spreading which leads to a chronic self-perpetuating process of organ inflammation and destruction (2), and (iv) the timing of MHC up-regulation in the target organ might be able to trigger the beginning of the process (3).

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Attempts to characterize autoantigenic epitopes in different autoimmune diseases have been made possible using random peptide phage libraries which allow the identification of ligands for disease-specific antibodies whether the antigen is known or not (4–7). This approach identifies linear, conformational as well as mimic epitopes, and it has been used to screen Ig fractions obtained from patients with multiple sclerosis (8), chronic immune thrombocytopenic purpura (9), type 1 diabetes mellitus (10), systemic lupus erythematosus (SLE) (11) and rheumatoid arthritis (RA) (12–14). One of the selected peptides identified by Ig from RA patients (12) showed homology with cereal glycine-rich cell wall proteins (GRP) and with the Epstein–Barr virus nuclear (EBV) antigen-I (EBNA-I). Analysis of GRP 1.8 (15), a ubiquitous protein found in French bean and related species, shows the presence of Gly–Ala repeated sequences homologous to EBNA-I and cytokeratins. Many studies have shown the ability of anti-EBNA-I antibodies to cross-react with different autoantigens such as heterogeneous nuclear ribonucleoprotein (hnRNP) (16), cytokeratin, collagen and actin in RA (17,18); the cross-reactions can be inhibited with synthetic peptides containing the Gly–Ala repeat sequence, implying that the cross-reactivity is due to anti-Gly–Ala antibodies that recognize both the host proteins and the EBNA-I repeat. A 15 amino acid synthetic peptide corresponding to amino acids 436–454 of GRP 1.8 and sharing homology with EBNA-I, fibrillar collagen and procollagen (Table 1) has been shown to induce humoral (14) and T cell responses in RA (19). Taken together, these data suggest that the autoimmune response in RA but also in other autoimmune diseases may not be as heterogeneous as originally thought. A possible consequence of such hypothesis is that the identification of widespread (auto)antigens may allow their use in the suppression of autoimmunity by oral administration. The suppression of autoimmunity by oral antigen administration may be a feasible therapeutic option (reviewed in 20) and the induction of oral tolerance can be a therapeutic strategy also in food allergy (reviewed in 21). Based on the observation that Gly–Ala repeated sequences are present in food proteins as well as in viruses and self-proteins, we decided to investigate the presence of anti-GRP antibodies in a large panel of sera from patients with autoimmune disorders. The 15 amino acid peptide derived from GRP was used to analyze human sera in a direct and competitive ELISA assay.

Serum IgG antibodies directed against such peptide were detected in different percentages of several autoimmune disorders and in food allergy. The GRP peptide was able to elicit a specific T cell response: peripheral mononuclear cells (PBMC) derived from patients with different diseases proliferated to the peptide and antigen-specific T cell clones could be generated from such patients. These data suggest that (auto)immune responses can be triggered by protein epitopes with crucial amino acids homologous to self proteins.

View this table: **Table 1.** Homology of GRP peptide with antigens

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Methods

Patients

The patients enrolled in the study attended the outpatient clinic of the Department of Clinical and Experimental Medicine, University of Verona, Italy. The patients affected by SLE and RA fulfilled the diagnostic criteria of the American College of Rheumatology (22,23). Psoriatic arthritis (PsA) was diagnosed by the presence of psoriasis and a seronegative peripheral arthritis, with or without axial skeletal involvement. Chronic idiopathic urticaria (CIU) was characterized by recurrent episodes of diffuse erythematous wheals with severe pruritus lasting for >6 weeks. Food allergy was identified by the presence of specific IgE and positive double-blind placebo-controlled challenge test; the offending foods were cereals, fruits and vegetables in 20 of 29 patients, fish in five of 29 and dairy products in four of 29 patients. Aeroallergy was assessed by a history of seasonal or perennial rhinitis and/or asthma and positive skin test for pollens or house dust mite. The patients with chronic parvovirus B19 infection (CPI) are described in details elsewhere (24). The number of the patients studied is reported in Table 2.

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View this table: **Table 2.** Patient's sera with IgG antibodies against GRP peptide

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The HLA status of patients with CPI has already been reported (24). In patients with PsA HLA DR4, DR1 and DR7 were found in three patients each; DR3, which is associated with a more aggressive form of the disease and the presence of erosive arthritis, was present in two patients. We did not find particular HLA class II antigens more frequently present in patients with SLE, CIU and food allergy, whereas in RA there was an increased prevalence of DR4 (30% of patients versus 15% of controls; $P = 0.008$). When the DR4 and DR1 were analysed at molecular level no difference was found in DR4 suballeles (*0401, *0402, *0403, *0404, *0405, *0408) between patients and controls; as far as DR1 suballeles were concerned (*0101, *0102, *0103) there was a statistical difference in the frequency of *0102 between the two groups (46% of patients versus 19% of controls; $P = 0.008$).

IgM rheumatoid factor was present in 25 of 32 RA patients and its level varied between 105 and 770 IU/ml (n.v. < 60 IU/ml).

All the patients' sera were tested for the presence of the following autoantibodies: anti-ssDNA antibodies, anti-keratin antibodies, anti-collagen II antibodies, anti-RNP antibodies. Anti-ssDNA antibodies were present in 37 of 37 SLE patients and in two of 32 RA patients, anti-keratin antibodies in seven of 32 RA patients and two in 12 of PsA patients, anti-collagen antibodies in eight of 32 RA patients and in one of 12 PsA patients, and anti-hnRNP antibodies in eight of 37 SLE patients. The sera of patients affected by CIU, food allergy and aero-allergy were negative for the autoantibody specificities studied.

Peptide synthesis and recombinant GRP proteins

The synthetic peptides (GRP peptide: GGYGDGGAHGGGYGG and the irrelevant control peptide: ALYPSSVGQPQFQGAP) were obtained by solid-phase synthesis using Fmoc-protected amino acids according to the method of Merrifield as modified by Atherton (25), and were purified by gel filtration. Recombinant GRP proteins with or without the sequence corresponding to the GRP peptide were expressed in *Escherichia coli* and purified according to Ryser *et al.* (15).

mAb and reagents

The purified mAb anti-CD56 (Leu19, IgG1), anti-CD8 (Leu2a, IgG1), anti-CD4 (Leu3a, IgG1), anti-CD3 (Leu4, IgG1) and anti-CD19 (Leu12, IgG1) were from Becton Dickinson (San Jose, CA). The purified mAb anti-CD16 (KD1, IgG2a) was a kind gift of Professor L. Moretta (Genoa, Italy). The class II specific mAb DC1-12 was a kind gift of Professor R. Accolla (Genoa, Italy). Phycoerythrin (PE)- or FITC-conjugated goat anti-mouse antisera were from Southern Biotechnology Associates (Birmingham, AL). Human keratin, collagen type II and actin were purchased from Sigma (St Louis, MO).

Affinity purification of IgG anti-peptide and anti-keratin antibodies

Serum IgG was obtained by affinity purification using a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden). The synthetic peptides or human keratin (5 mg antigen/g dried Sepharose powder) were coupled to Sepharose CL4B (Pharmacia), according to the manufacturer's instructions. Affinity-purified IgG samples diluted in PBS were applied to the column. Bound IgG was eluted with 0.1 M glycine (pH 2.5) and dialyzed against PBS.

ELISA assay

The synthetic peptides and the recombinant proteins were used at a concentration of 20 µg/ml in PBS to coat polystyrene plates (Nunc, Roskilde, Denmark). After blocking with 5% dry non-fat milk in PBS, the antibodies diluted in 2.5% dry non-fat milk and 0.05% Tween in PBS were added and incubated for 4 h. The plates were then washed and alkaline phosphatase-conjugated goat anti-human IgG (F(ab')₂ fragment) or anti-mouse IgG or IgM (Sigma) were added and incubated overnight at 4°C. After washings, the bound enzymatic activity was measured with *p*-nitrophenylphosphate (Sigma). Each antibody preparation was tested on a control plate not coated with the antigen. This non-specific binding never exceeded 10% of the specific binding (e.g. to the antigen coated plate). For competitive assays the amount of antibody that gave 50% of the maximum binding to the antigen on

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the solid phase was preincubated with different amounts of competitors or buffer for 1 h at 37°C and then transferred to the antigen-coated plates. The assay was then carried on as the direct binding assay. In the ELISA assay for the detection of serum antibodies directed against the peptide or the recombinant GRP protein containing the peptide sequence of interest, 25 sera diluted 1: 100 from normal age- and sex-matched subjects were used as control group. Optical density values higher than the mean + 3 SD of each serum dilution of the control group (OD > 70 for the GRP peptide and OD > 85 for the recombinant protein) were considered positive.

The direct and competitive ELISA for human keratin, collagen type II and actin has been described (24). The ELISA assay for EBNA-I was performed using a commercially available kit (Sigma).

Immunization of BALB/c mice

The synthetic peptide was coupled to the carrier protein keyhole limpet hemocyanin (KLH) and emulsified in Freund's adjuvant. Mice were injected 5 times (the first time in complete Freund's adjuvant, the other times in incomplete adjuvant) at the base of the tail at 15 day intervals. The animals were bled 7 days after the last injection and the sera tested in ELISA. Control animals were injected with adjuvant alone, keyhole limpet hemocyanin (KLH) alone or coupled with the irrelevant peptide.

Generation of CD4⁺-specific T cells clones and flow cytometric analysis

PBMC derived from patients were isolated on Ficoll-Hypaque gradient and cells were then incubated with peptide at 20 µg/ml in 96 U-bottomed microplates in complete medium. After 10 days of culture in absence of rIL-2 the cells were incubated with a mixture of anti-CD16 (KD1), anti-CD56 (Leu19) and anti-CD8 (Leu2a) mAb and purified by immunodepletion using goat anti-mouse Ig coated with magnetic beads (Unipath, Milan, Italy) (26). Viable cells were cloned under limiting dilution in the presence of irradiated peripheral blood lymphocytes as feeder cells in complete medium and of exogenous rIL-2 (Cetus, Emeryville, CA) as described for T cell cloning (27).

Cells were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse Ig (28); control aliquots were stained with the fluoresceinated reagent alone. All samples were analyzed on a flow cytometer FACSort (Becton Dickinson) gated to exclude non-viable T cells.

Human mAb

Epstein-Barr virus-transformed cell lines were derived from selected individuals according to standard procedures (29) and were cloned in soft agar; the resulting Ig-producing clones were tested for antigen-binding activity by ELISA assay.

Proliferation assay

PBMC or highly purified CD4⁺ clones were cultured for 3 days in complete medium in 96-well U-bottom microplates (5×10^5 cells/well) with irradiated autologous PBMC or B-EBV-transformed cell lines (5×10^5 cells/well). The following stimuli were used: GRP peptide (20 µg/ml), an irrelevant peptide (20 µg/ml) and rIL-2 (20 U/ml) as positive control. Cells were then pulsed with 20 µCi [³H]thymidine and incubated for an additional 18 h at 37°C. Results are expressed in c.p.m. $\times 10^{-3}$ of the mean ± SD of triplicate samples of two different tests for each patient.

Identification of the phenotype of individual clones.

The T_h subsets of the clones obtained was analyzed by flow cytometric assessment of intracytoplasmic cytokine content (reviewed in 30). Cells were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (Sigma) plus 1 µg/ml ionomycin (Sigma) for 4 h in the presence of 10 µg/ml Brefeldin A (Sigma) (31) and were subsequently fixed with PBS containing 4% (v/v) paraformaldehyde and permeabilized in PBS/saponin buffer (Sigma). Directly conjugated monoclonal anti-cytokine antibodies specific for IL-2, IFN-γ, 1L-4, IL-5 and IL-10 were used to identify the cytokines produced [FITC-conjugated anti-IFN-γ and R-PE-conjugated anti-IL-2 and -IL-4, from Becton Dickinson; R-PE-anti-IL-5 and -IL-10, from PharMingen (San Diego, CA)]. Specificity controls were performed using isotypical mAb (IgG2a-FITC and IgG1-R-PE, both from Becton Dickinson). At least 30,000 events were acquired by FACScan flow cytometer equipped with an argon ion laser (488 nm) and CellQuest software (Becton Dickinson).

► Results

Patients' sera recognize the synthetic peptide.

A peptide immunoselected from a phage display library using a pool of Ig from RA patients showed interesting homologies with a food-derived antigen, viral-encoded proteins and autoantigens (Table 1) (19). Based on the high homology between the peptide derived from GRP and common autoantigen targets we synthesized and used the GRP peptide to screen a large number of patients' sera affected by either autoimmune or allergic diseases as well as normal donors. Table 2 shows the results of the ELISA assay performed in the patients studied: the frequency of patients' sera able to recognize the GRP peptide was particularly high in certain diseases such as PsA, CIU and SLE. A large number (15 of 29) of sera from patients with food allergy also reacted with the peptide; on the contrary, none of the 21 patients with aeroallergy and of normal donors recognized the peptide. The results were confirmed in each patient's serum by competitive immunoassays where the peptide in liquid-phase competition experiments was shown to be able to displace the binding of serum IgG from the peptide on the solid phase (Fig. 1A).

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Fig. 1. (A) Inhibition of binding of serum IgG antibodies to solid phase GRP peptide. The serum (from patient LM) was preincubated with different amounts of antigen (GRP peptide and control irrelevant peptide, µg/ml in diluting buffer) for 1 h at 37 ° C and then transferred to a GRP peptide-coated plate. Bound antibodies were detected by an alkaline phosphatase-labeled anti-human IgG antiserum. Results are expressed as percent inhibition. Inhibition experiments were performed in all the patients included in the study. The mean of the amount of liquid-phase inhibitor required to obtain 50% inhibition of the binding of serum Ig to the solid-phase antigen in the competitive assay is 4 ± 1.2. (B) Inhibition of binding of serum IgG antibodies to solid-phase GRP recombinant protein. The serum (from patient VM) was preincubated with different amount of antigens [GRP

recombinant protein (GST1fGRPC), GRP peptide and control irrelevant peptide, µg/ml in diluting buffer] for 1 h at 37°C and then transferred to a plate coated with GRP recombinant protein. Bound antibodies were detected by an alkaline phosphatase-labeled anti-human IgG antiserum. Results are expressed as percent inhibition. The other 14 patients' sera had a similar behavior. The means of liquid-phase inhibitors required to achieve a 50% inhibition of the binding of serum Ig to the solid-phase GRP recombinant protein are: (i) GRP peptide: 10 ± 1.2 and (ii) GRP recombinant protein: 8 ± 1.1 .

Anti-GRP IgA antibodies were detected in two of 32 RA patients, in four of 37 SLE patients, in none of seven CPI patients, in one of 12 PsA patients, in three of 12 CIU patients and in 11 of 29 food allergy patients.

Anti-GRP IgE antibodies were not detected in any patients' group.

These data suggest that anti-GRP peptide IgG antibodies are widely present in the serum of patients with autoimmune disorders and with food allergy.

Serum anti-peptide antibodies specifically recognize the native recombinant protein

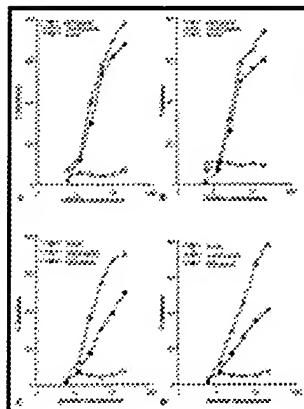
Prokaryotic expression plasmids encoding truncated forms of the GRP were expressed in *E. coli* (15). SDS-PAGE analysis of lysates of *E. coli* expressing these truncated forms demonstrated that each lysate contained an additional protein of the expected molecular mass. The different recombinant proteins were then purified and used in an ELISA assay to confirm the reactivity of the sera with the peptide. Fifteen patients' sera containing serum antibodies against the GRP peptide as well as normal controls' sera were tested on the various forms of recombinant proteins. All the 15 sera reacted with the recombinant protein (GST1fGRPC) containing the insert corresponding to the GRP peptide sequence, but not with the other truncated versions of the molecule. Similarly sera which did not recognize the GRP peptide did not react with the recombinant protein. Normal human sera did not recognize the recombinant protein (data not shown). In a competitive immunoassay the liquid-phase GRP peptide could displace the binding of serum Ig from the recombinant protein on the solid phase (Fig. 1B), further confirming the specificity of such antibody interaction. The data obtained indicate that serum antibodies are able to recognize the GRP peptide sequence even in the context of a larger molecule such as the GRP recombinant protein. This observation is important because it may constitute the basis for a wide cross-reactivity of antibodies directed against the GRP peptide.

Serum anti-peptide antibodies cross-react with other autoantigens

To further characterize the fine specificity of the binding of serum anti-GRP peptide antibodies we isolated the IgG anti-peptide component from the sera of five different patients by affinity chromatography using a peptide-Sepharose column. Anti-GRP peptide antibodies affinity purified from five normal donors were used as control. Anti-GRP peptide antibodies isolated from the patients' sera recognized the peptide in both direct and competitive ELISA, and they also reacted with the recombinant protein (data not shown).

These antibody preparations were then tested for their ability to recognize other autoantigens such as human keratin, collagen type II, actin, EBNA-I. As shown in Table 3, the anti-GRP peptide reacted with different autoantigens and the affinity of the interaction varied within the different serum samples. On the contrary anti-GRP peptide antibodies purified from the serum of normal subjects showed a low-affinity interaction with the GRP peptide and did not cross-react with any of the autoantigens tested. Moreover 20 times more serum was needed to purify the same amount of anti-peptide Ig when compared to the patients' sera, indicating that such anti-peptide antibody population was much less represented in the sera of the healthy donors as compared to the patients studied. Figure 2(A and B) shows a cross-inhibition experiment in which the binding of two anti-GRP peptide antibody preparations to solid-phase keratin is cross-inhibited by the GRP peptide but not by an irrelevant control peptide.

View this table: **Table 3.** Autoantigen binding properties of anti-GRP peptide antibodies
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Fig. 2. Inhibition of binding of affinity-purified anti-GRP peptide antibodies to keratin (A and B). Affinity-purified anti-GRP peptide antibodies [from patient GC (A) and patient CA (B)] were preincubated with different amount of antigens (keratin, GRP peptide and control irrelevant peptide, $\mu\text{g}/\text{ml}$ in diluting buffer) for 1 h at 37°C and then transferred to a plate coated with keratin. Bound antibodies were detected by an alkaline phosphatase-labeled anti-human IgG antiserum. Results are expressed as percent inhibition. Inhibition of binding of affinity-purified anti-keratin antibodies to solid-phase keratin (C and D). Affinity-purified anti-keratin antibodies [from patient GC (C) and patient SP (D)] were preincubated with different amount of antigens (keratin, GRP peptide and control irrelevant peptide, $\mu\text{g}/\text{ml}$ in diluting buffer) for 1 h at 37°C and then transferred to a keratin-coated plate. Bound antibodies were detected by an alkaline phosphatase-labeled anti-human IgG antiserum. Results are expressed as percent inhibition.

To further assess the specificity of the autoantigen binding activity of the anti-GRP antibodies isolated from the patients' sera the irrelevant control peptide was used to affinity purify anti-peptide antibodies from the same five patients. Such anti-control peptide antibodies were then tested for their ability to cross-react with autoantigens (collagen II, keratin, actin and EBNA-I). None of the five anti-peptide antibody preparations reacted with the autoantigens studied (data not shown).

These results indicate that anti-GRP peptide antibodies isolated from the sera of autoimmune patients are able to cross-react with autoantigens including keratin, collagen and EBNA-I. This cross-reactivity is not shown by anti-control peptide antibodies obtained from the same subjects.

In a separate set of experiments anti-keratin antibodies were affinity purified from the serum of patients GC and SP using a keratin-Sepharose column. The purified antibody preparations recognized human keratin in direct and competitive ELISA (data not shown). Such anti-keratin antibody populations were used to perform a cross-inhibition experiments in which the binding of these antibodies to solid-phase keratin is competed by keratin and GRP peptide. As shown in Fig. 2(C and D), the binding of these antibodies to keratin is completely inhibited by keratin and only partially by GRP peptide. These data indicate that anti-GRP antibodies are able to cross-react with autoantigens (keratin, actin, EBNA-I), whereas not all the antibodies directed against such autoantigens are able to recognize the GRP sequence.

These results indicate that the peptide studied identifies a shared epitope widely expressed in different diseases and that antibodies against such peptide recognize different autoantigen targets.

EBV-transformed cell clones producing anti-GRP antibodies

Six EBV-transformed cell clones obtained from patients PG, GC and SP produced anti-GRP peptide antibodies able to recognize keratin, actin and EBNA-I. An example of this behavior is given in Fig. 3. These results confirm at the clonal level our previous observations that anti-GRP antibodies produced in subjects with autoimmune disorders are able to cross-react with several autoantigens and this cross-reactivity may be relevant in the pathogenesis of the disease.



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Fig. 3. Autoantigen binding activity of a human monoclonal anti-GRP peptide antibody. (A) Direct binding of mAb 1K1 to different antigens: keratin, actin, EBNA-I, GRP peptide and control peptide. Bound antibodies were detected by an alkaline phosphatase-labeled anti-human antiserum. Results are expressed as absorbance at 405 nm, 30 minutes after substrate addition. (B) Inhibition of binding of human mAb 1K1 to solid-phase GRP peptide. The amount of purified Ig which gave 50% of the maximal binding to the antigen in direct ELISA was preincubated for 1 h at 37° C with different amount of antigens (keratin, actin, EBNA-I, GRP peptide and control peptide) and then transferred to the GRP peptide-coated plate. Bound antibodies were detected by an alkaline phosphatase-labeled anti-human antiserum. Results are expressed as percent inhibition.

Normal mice immunized with the GRP peptide develop an autoimmune response

To further substantiate the role of the GRP peptide in inducing an autoimmune response, 10 BALB/c mice were immunized with the GRP peptide coupled to the carrier protein KLH. Eight mice developed an anti-GRP peptide IgG and IgM response (Table 4). Autoantibody activity against keratin, collagen II and EBNA-I was detected in all the mice which developed a strong anti-GRP response, and anti-peptide antibodies affinity purified from the pooled sera of five individual mice recognized the autoantigens as well (data not shown). On the contrary, autoantibody activity could not be detected in the sera of the animals injected with an irrelevant peptide coupled to KLH, KLH alone or PBS alone. These data further indicate that the GRP peptide is able to induce an autoimmune response through

View this table: **Table 4.** Autoantibody response in mice injected with GRP peptide
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Generation of peptide-specific T cell clones

To evaluate the T cell response to the GRP peptide, 11 patients affected by different diseases were selected on the basis of a high anti-peptide serum IgG antibody titer. The 11 patients selected for the preparation of T cell clones were affected by the following diseases: food allergy (patient CA), SLE (patients LoM, PG, ZP), PsA (patients LM and SP), RA (patients VM and GC) and CPI (patients BS, CoL, SE). PBMC isolated from these patients were able to proliferate to the GRP peptide, but not to a control irrelevant peptide, whereas normal subjects did not show any proliferative response to the GRP peptide (data not shown).

One hundred and fifty antigen-specific T cell clones were derived from the PBMC of the 11 patients studied. The clonal efficiency of the clonal procedures was 4.82% ($SD \pm 1.1$). We then chose to characterize 38 clones on the basis of their growth capability; all the clones proved to be $CD4^+$ by FACS analysis. The antigen specificity of the 38 clones was then evaluated using the GRP peptide and EBV-transformed autologous B lymphocytes or autologous PBMC as antigen-presenting cells. Twenty-five T cell clones showed a specific proliferative response to the GRP peptide, but not to an irrelevant control peptide. Of the 25 peptide-specific T cell clones, six were derived from patient SP, four from patient CA, two from patients SE, CG, VM, LM, PG, LoM, one from each of ZP, CoL, BS. The specificity of the proliferative response of the $CD4^+$ T cell clones obtained from the patients studied was confirmed by the following lines of evidence: (i) all the anti-GRP T cell clones did not proliferate in the presence of the GRP peptide when heterologous irradiated EBV cells or heterologous PBMC were used as antigen-presenting cells and (ii) preincubation of the $CD4^+$ anti-GRP peptide clones with the class II-specific mAb D1-12 abolished the proliferative response of these clones to the GRP peptide (data not shown). Figure 4 shows the proliferative response of representative clones derived from different patients.

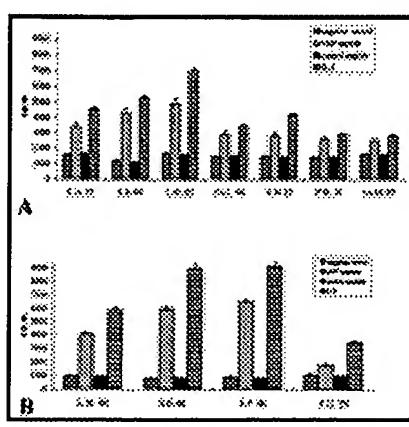


Fig. 4. Proliferative responses of GRP peptide-specific T cell clones. Proliferation of antigen-specific T-cell clones derived from different individual patients. $CD4^+$ clones derived were cultured in complete medium in 96-well U-bottom microplates (5×10^5 cells/well) with irradiated autologous PBMC or autologous B-EBV-transformed cell lines derived from patients (5×10^5 cells/well). The following stimuli were used: GRP peptide (20 μ g/ml), an irrelevant peptide (20 μ g/ml) and rIL-2 (20 U/ml) as positive control. After 3 days of culture cells were pulsed with 20 μ Ci [3 H]thymidine and incubated for an additional 18 h at 37°C. Results are expressed in c.p.m. $\times 10^{-3}$ of the mean \pm SD of

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triplicate samples of two different tests for each patient's clone. The antigen-presenting cells used are: autologous PBMC (A) and autologous B EBV-transformed cell lines (B). Statistical analysis of the data was performed using the paired *t*-test; the differences between the proliferative responses of T cell clones to the GRP peptide and to the control peptide were statistically significant ($P < 0.001$).

PBMC obtained from two normal subjects were stimulated with the GRP peptide and subsequently cloned with the previously described procedure: differently from what was observed with the clones derived from the patients' PBMC, the CD4⁺ T cell clones obtained from the two normal subjects failed to proliferate to the GRP peptide (data not shown), further confirming the specificity of the anti-GRP T cell response observed in the group of patients examined.

Taken together, these results suggest that a specific T cell response against the GRP peptide can be elicited in patients affected by different autoimmune diseases.

Intracytoplasmic cytokine profile of the T cell clones studied

In order to evaluate the T_h subset of the peptide-specific T cell clones, we performed intracellular cytokine staining by FACS analysis. The 18 clones analyzed did not show a T_h1 or T_h2 cytokine profile, since IFN- γ , IL-2 and IL-4 were simultaneously present (data not shown). These data suggest that GRP peptide-specific T cell clones belong to the T_h0 subset.

► Discussion

This study shows that a peptide from a food-derived glycine-rich cell wall protein is able to elicit a B and T cell immune response in several autoimmune diseases. Such peptide has a high homology with EBNA-I, cytokeratins, and other self antigens such as hnRNP, fibrillar collagen and procollagen (19). Interestingly the reactivity to this peptide is not limited to a particular disease condition since anti-peptide IgG antibodies and peptide-specific T cell clones can be found in several autoimmune disorders and in food allergy, whereas normal subjects do not have detectable serum antibodies directed against the peptide and their PBMC fail to proliferate to the peptide. The anti-GRP peptide antibodies purified from the sera of the patients studied were able to specifically recognize EBNA-I and different autoantigen targets (keratin, collagen, actin), whereas such autoantigen binding activity was not displayed by the anti-control peptide antibodies affinity purified from the same patients' sera and by the anti-GRP peptide antibodies eluted from the sera of healthy donors. In the latter case the eluted antibodies showed a low-affinity interaction with the peptide and did not recognize any of the autoantigens tested. The autoantigen binding profile of the anti-GRP peptide antibodies is similar to the one observed with serum antibodies directed against P62, a synthetic peptide corresponding to the Gly-Ala repeat sequence of EBNA-I (18). In both cases affinity purified anti-peptide antibodies cross-reacted with the same autoantigens: keratin, actin and collagen. The similar behavior of the two anti-peptide antibody populations (anti-P62 and anti-GRP peptide) can be ascribed to the presence

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of the common sequence Gly–Gly–Ala, which suggests that anti–Gly–Ala antibodies may be responsible for the cross–reactions observed. An increased level of anti–Gly–Ala antibodies may account for the cross–reaction with structural endogenous proteins such as cytokeratins and collagen. In this regard, recent data obtained in our laboratory using recombinant peptide libraries show that the Gly–Gly–Ala motif is recognized at high frequency in randomly generated dodecamer peptides by autoantibodies belonging to different autoimmune diseases. This finding further demonstrates an important role for this amino acid sequence as an antigen target in different diseases (Lunardi *et al.*, manuscript in preparation).

The finding that anti–GRP IgG antibodies are present at high frequency in the sera of autoimmune and food allergic individuals was further confirmed by the generation of human mAb from EBV–transformed lymphocytes belonging to representative patients. The anti–GRP peptide reactivity was present at high frequency in a panel of randomly selected EBV clones. Similarly to the serum counterpart, these antibodies were able to cross–react with several autoantigens. These results again support an important role for the GRP sequence in priming an autoimmune response. This hypothesis was further substantiated by experiments in which the 15 amino acid GRP peptide was coupled to a carrier protein and its ability to elicit an autoimmune response in normal animals (BALB/c mice) was analyzed. Interestingly mice displaying a strong anti–peptide response produced antibodies able to bind the GRP peptide, and to cross–react with several autoantigens including keratin, collagen II and EBNA–I.

In accordance to the humoral response, T cell responses can be elicited by the GRP peptide in different patients and in several disease conditions. A T cell response against the GRP peptide has been already described in the synovial fluid lymphocytes of patients with juvenile RA and GRP peptide specific CD4⁺ T cell clones have been obtained from the synovial fluid of these patients (19). Interestingly the GRP peptide is able to induce proliferation of PBMC isolated from patients with different diseases, whereas normal subjects do not proliferate to the peptide.

To further analyze the T cell response to the peptide, a panel of CD4⁺ peptide–specific T cell clones was derived from PBMC of patients with different disease conditions. CD4⁺ T cell clones specific for the GRP peptide could not be derived from the PBMC of normal healthy donors. Peptide–specific CD4⁺ T cell clones may either be the consequence of a previous activation and expansion by exogenous antigens, e.g. in the gut mucosa or more likely derive from normally present autoreactive T cell subsets (3). Independently from the mechanism involved in the generation of such CD4⁺ T cell clones, these cells may be able to provide a T cell help to B cells for the generation of an IgG response towards the peptide, which will result in the production of large amounts of cross–reacting anti–peptide antibodies. The presence of the same structural motif in the different autoantigens may account for the promiscuity of such autoantibody response which seems to be a common feature of the immune response in different autoimmune disorders. These results suggest that the immune response in autoimmune diseases may not be as heterogeneous as originally thought. In this regard it is possible to view the autoimmune response as an oligoclonal expansion of a rather limited number of T cell subsets which share the capability of responding to a particular amino acid sequence. The process may result in the stimulation of cells able to deliver T cell help to a great number of B cell

Glycine-rich cell wall proteins act as specific antigen targets in autoimmune and food... Page 13 of 17 clones. Thus, a large number of antibodies specific for different autoantigenic targets may arise as a response to a relatively small number of amino acid sequences, probably due to a mechanism of molecular mimicry.

Interestingly, a B and T cell response to the GRP peptide can also be found in patients with food allergy. The absence of anti-GRP-specific IgE antibodies in patients with food allergy suggests the possibility of a non-IgE-mediated immune response to particular food-derived antigens which can be able either to block or to mediate histamine release from basophils and mast cells.

The type of immune response elicited may be determined by the genetic background: the TCR-peptide-MHC interaction can control the direction of the functional immune response, and MHC linkage to polarized T_h 1-type and T_h 2-type immune responses has now been reported for several antigens and peptides (32–38). The analysis of some of our peptide-specific T cell clones derived from peripheral blood of patients with different diseases revealed a predominant T_h 0 phenotype. We can hypothesize either that the GRP peptide is unable to induce a dominant T_h 1 or T_h 2 cytokine response, or that this peptide-specific CD4 $^+$ subset found in the peripheral blood may switch to a T_h 1 or T_h 2 cytokine response at sites of autoimmune inflammation.

Several lines of evidence suggest that the induction of oral tolerance by orally administered antigens has potential therapeutic applications for the treatment of autoimmune and food-allergic diseases (20,21). In this regard the finding that the same peptide epitope can induce an immune response in different diseases may have important practical implications. The identification of widespread peptide antigens may suggest their potential utilization in the suppression of the immune response by oral administration. This therapeutic strategy has so far been hampered mainly by the limited knowledge of disease relevant antigens.

Random peptide library technology is a powerful tool for identifying potentially pathologically relevant peptide antigens in different disease conditions. Using this approach it is possible to dissect common features at the amino acid level in individuals affected by the same or by different diseases. This could allow the identification of peptide sequences recognized at high frequency in certain conditions: the GRP peptide represents an example of an antigenic peptide sequence able to prime a B and T cell immune response in different and apparently unrelated diseases.

Finally, the finding of a common peptide epitope able to elicit an immune response in patients with food allergy and different autoimmune disorders give rise to the question of a possible link between food antigens, gut mucosa and systemic immune response. In the past few years several groups have studied the role of dietary manipulation in RA (39–41), juvenile RA (42) and vasculitis (43), reaching conflicting results. T cell clones specific for particular food antigen epitopes may arise in the gut mucosa and be recruited to particular sites, such as joints, where they proliferate in response to homologous peptides derived from synovial proteins, following local inflammation and up-regulation of MHC molecules. The release of additional self-antigens and/or epitope spreading can lead to a chronic self-perpetuating process of organ inflammation and destruction.

In conclusion, our data suggest that phylogenetically highly conserved epitopes in plants, viruses and humans may be responsible for an autoimmune response in susceptible individuals.

► Acknowledgments

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► Abbreviations

CIU chronic idiopathic urticaria

CPI chronic parvovirus infection

EBNA-I Epstein-Barr nuclear antigen I

EBV Epstein-Barr virus

GRP glycine-rich cell wall protein

hnRNP heterogeneous nuclear ribonucleoprotein

KLH keyhole limpet hemocyanin

PBMC peripheral blood mononuclear cells

PE phycoerythrin

PsA psoriatic arthritis

RA rheumatoid arthritis

SLE systemic lupus erythematosus

► Notes

The first two authors contributed equally to this work

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INHIBITION OF CHRONIC REJECTION OF AORTIC ALLOGRAFTS BY DIETARY GLYCINE.

Transplantation. 69(5):773-781, March 15, 2000.

Yin, Ming 1; Rusyn, Ivan 1; Schoonhoven, Robert 2; Graves, Lee M. 1; Rusyn, Elena V. 3; Li, Xiangli 1; Li, Fengling 4; Cox, Adrienne D. 1 3; Harding, Tim W. 5; Bunzendahl, Hartwig 6; Swenberg, James A. 2; Thurman, Ronald G. 1

Abstract:

Background. Chronic rejection is influenced by a variety of risk factors, including histoincompatibility and ischemia. Glycine, a cytoprotective agent, has been shown to protect against ischemia-reperfusion injury in the liver, inactivate hepatic resident macrophages, minimize cyclosporin A-induced nephrotoxicity, and exhibit immunosuppressive properties in vitro. The aim of this study was to investigate whether dietary glycine could reduce development of chronic rejection.

Methods. Lewis recipients of Fisher-344 abdominal aortic allografts received diets that contained either 5% glycine plus 15% casein or 20% casein as control for 10 weeks. Vascular lesions of aortic isografts and allografts were evaluated quantitatively with image analysis and cell counting.

Results. No significant vascular changes were observed in isografts (mean medial areas of $3.3+/-0.3 \times 10^5 [\mu\text{m}^2]$). However, dramatic intimal thickening (neointimal area $2.1+/-0.3$) and medial thinning ($1.5+/-0.3$) were observed in allografts from rats fed control diet. In contrast, glycine significantly reduced the neointima by 45% ($1.2+/-0.3$) and protected the media ($3.5+/-0.2$). This led to intima to media area ratios almost twice as large in the control group as in glycine-fed rats ($2.2+/-0.4$ vs. $1.1+/-0.3$, $P <0.05$). Moreover, infiltrating leukocytes, especially macrophages, were reduced significantly in the adventitia by glycine. In addition, glycine inhibited proliferation and migration of rat aortic smooth muscle cells in culture by 45 and 60%, respectively.

Conclusion. These results indicate that dietary glycine minimizes histopathological changes of chronic rejection by reducing the immune response and, in part, by minimizing proliferation and migration of smooth muscle cells.

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INHIBITION OF CHRONIC REJECTION OF AORTIC ALLOGRAFTS BY DIETARY GLYCINE

Yin, Ming¹ 7; Rusyn, Ivan¹; Schoonhoven, Robert²; Graves, Lee M.¹; Rusyn, Elena V.³; Li, Xiangli¹; Li, Fengling⁴; Cox, Adrienne D.¹ 3; Harding, Tim W.⁵; Bunzendahl, Hartwig⁶; Swenberg, James A.²; Thurman, Ronald G.¹

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Abstract TOP

Background. Chronic rejection is influenced by a variety of risk factors, including histoincompatibility and ischemia. Glycine, a cytoprotective agent, has been shown to protect against ischemia-reperfusion injury in the liver, inactivate hepatic resident macrophages, minimize cyclosporin A-induced nephrotoxicity, and exhibit immunosuppressive properties *in vitro*. The aim of this study was to investigate whether dietary glycine could reduce development of chronic rejection.

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(mean medial areas of $3.3 \pm 0.3 \times 10^5 \mu\text{m}^2$). However, dramatic intimal thickening (neointimal area 2.1 ± 0.3) and medial thinning (1.5 ± 0.3) were observed in allografts from rats fed control diet. In contrast, glycine significantly reduced the neointima by 45% (1.2 ± 0.3) and protected the media (3.5 ± 0.2). This led to intima to media area ratios almost twice as large in the control group as in glycine-fed rats (2.2 ± 0.4 vs. 1.1 ± 0.3 , $P < 0.05$). Moreover, infiltrating leukocytes, especially macrophages, were reduced significantly in the adventitia by glycine. In addition, glycine inhibited proliferation and migration of rat aortic smooth muscle cells in culture by 45 and 60%, respectively.

Conclusion. These results indicate that dietary glycine minimizes histopathological changes of chronic rejection by reducing the immune response and, in part, by minimizing proliferation and migration of smooth muscle cells.

Chronic rejection, or transplant allograft vasculopathy, has been identified as the most important cause of renal graft failure after the first posttransplant year (1), and is a major factor affecting long-term survival of transplanted organs (2). Vascular changes due to intimal hyperplasia, an important characteristic of this process, constitute the morphological hallmark of chronic rejection (3, 4). The proliferative vascular changes in chronic rejection are similar in all transplanted solid organs (5), and there is no effective treatment. In cardiac allografts undergoing chronic rejection, concentric vascular intimal thickening occurs together with medial thinning, interstitial fibrosis and perivascular infiltration of mononuclear cells (5, 6). Intimal hyperplasia involves proliferation of transformed secretory type smooth muscle cells and infiltration of inflammatory macrophages and activated T lymphocytes (5). Transplant vasculopathy affects the entire length of the vessels including major vessels as well as the small penetrating intramyocardial branches in the heart (7). Congestive heart failure, ventricular arrhythmia, myocardial infarction, and sudden death may occur.

Although the exact pathogenesis of chronic rejection remains to be established, several risk factors, some dependent and some independent of allogenic immune responses, have been identified (8). Long-term graft survival of HLA identical sibling renal transplants in humans and experimental syngeneic transplants in inbred animals support the role of host immunological responsiveness in the process (9). The frequency and intensity of acute rejection episodes are also associated with an increase in the prevalence and severity of chronic rejection (10). However, the influence of factors independent of immune responses has attracted increasing interest (11). Potential risk factors include early injury secondary to prolonged ischemia and reperfusion, cytomegalovirus infection, disorders of lipoprotein metabolism and the impact of immunosuppressive drugs (3, 8). Cyclosporin A- (CsA) induced nephrotoxicity, hypercholesterolemia and hypertension may ultimately worsen or accelerate the chronic process of graft loss (8). Moreover, some of these factors, such as ischemia-reperfusion injury and infection, may be confounding variables contributing to the disease process (12). Therefore, to improve long-term survival, the primary strategy is to avoid known risk factors and to immunosuppress the recipient sufficiently although avoiding overimmunosuppression.

Glycine, a cytoprotective agent and immunomodulatory amino acid, has been shown to protect against hypoxia-reoxygenation injury in the liver (13), inactivate hepatic resident macrophages (14), and exhibit immunosuppressive properties in vitro (15). Moreover, diets supplemented with glycine, a simple long-term dietary therapy, minimize cyclosporin A-induced nephrotoxicity, inhibit hepatocyte proliferation caused by peroxisome proliferators, and improve survival in endotoxin shock in rats (14, 16, 17). These findings led to the hypothesis that dietary glycine would minimize chronic allograft rejection. Therefore, our study was designed to test this hypothesis using a rat aortic transplant model. Indeed, a glycine-containing diet inhibited allograft vasculopathy by reducing intimal thickening and medial thinning in vivo and suppressed smooth muscle cell proliferation in vitro. Preliminary accounts of this work have appeared elsewhere (18).

MATERIALS AND METHODS TOP

Animals and diets. TOP

Two inbred strains of female rats weighing 200 to 240 g with minor histocompatibility differences were chosen for these studies: Fisher-344 and Lewis. Allogenic transplantations were performed from Fisher-344 donors to Lewis recipients ($n=10$ per group), and syngeneic transplantations (i.e., isografts, $n=4$ per group) were from Lewis to Lewis rats. The animals were

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housed in an AALAC-approved facility for one week before surgery with free access to standard laboratory chow (Agway PROLAB RMH 3000, Syracuse, NY) and tap water. Two days before transplantation, recipient rats were randomly assigned to two experimental groups and fed a semisynthetic powdered diet (AIN-93G (19), Teklad test diets, Madison, WI) containing 5% glycine and 15% casein (glycine group) or 20% casein (control group). After transplantation, each recipient rat continued to receive its assigned diet throughout the entire experimental period of 10 weeks. All animals received humane care in compliance with guidelines approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Aortic transplantation. TOP

Rats were anesthetized by inhalation of methoxyflurane (Mallinckrodt Veterinary, Inc., Mundelein, IL). The abdominal aorta of each recipient rat was dissected free below the left renal arteries to the bifurcation of the abdominal aorta, as described previously (20). A 14- to 18-mm long segment of the aorta was removed from the donor rat and stored at 4°C in lactated Ringer's solution (Baxter, Deerfield, IL) during preparation of the recipient. Each graft was immediately transplanted orthotopically by an end-to-end anastomosis with 8-0 nylon sutures (Ethicon, Somerville, NJ). The aortic clamps were then removed and patency of the new vascular connection was confirmed. Average cold ischemia time was about 35 min, and no pharmacological immunosuppression was used. Ten weeks after transplantation, recipient animals were anesthetized and the aortic grafts were excised. Each graft was divided into four parts. Two parts were fixed in 4% buffered formaldehyde and embedded in paraffin, and the other two parts were frozen and stored at -80°C until processed for immunohistochemistry.

Measurement of glycine concentration in blood. TOP

Blood samples were collected at necropsy and serum was stored at -80°C until measurement. Concentrations of glycine in serum were determined as described by Ohmori (21). Briefly, glycine was extracted, benzoylated, and the resulting hippuric acid was extracted and dried. Subsequently, the concentration of a colored conjugate of hippuric acid was determined spectrophotometrically at 458 nm.

Image analysis of rat aortic grafts. TOP

A minimum of two cross-sections (5 µm thickness) of each isograft or allograft was taken from each animal, embedded in paraffin, and stained with elastic-van Gieson and hematoxylin. The cross-sectional areas of intima and media of the aortic allografts were measured using a BioQuant TCW95 image acquisition and analysis system (Nashville, TN) incorporating an Olympus BH2 microscope (Olympus America Inc., Lake Success, NY). The medial area was defined as the area between the external and internal elastic lamina. The neointimal area was defined as the area between the internal lamina and arterial lumen (22).

Histology. TOP

Paraffin-embedded isografts and allografts were cut into 5-µm thick cross-sections and stained with hematoxylin and eosin. Evaluation of grafts was performed semiquantitatively by scoring the severity of vascular changes (intimal thickening, medial thinning and adventitia inflammation) from 0 to 3 (0=no changes, 1=mild, 2=moderate, and 3=severe). Sections were scored by an outside investigator without knowledge of group assignment. The number of nuclei in a given anatomical area using straight, cross-sectional lines and a 10-mm grid in the eye-piece was counted in four random areas of the adventitia layer of each graft (magnification ×200), and means were calculated. It has been shown that nuclei in the allograft adventitia are predominantly inflammatory infiltrates (23, 24).

Immunohistochemical staining. TOP

Five-µm thick cross-sections of paraffin-embedded grafts were stained for detection of monocyte/macrophages (ED1⁺ cells). In addition, 5-µm thick cross-sections of frozen sections were stained for pan T-lymphocytes (CD5⁺ cells) and proliferation cell nuclear antigen (PCNA) using the Dako Envision System. Primary antibodies against ED1 and CD5 were purchased from Biosource International (Camarillo, CA). Immunohistochemistry was quantitated by counting the number of positive-stained cells using the above-mentioned eye-piece with a magnification of 400×. Four areas per section were randomly selected and counted. Data were pooled to determine means.

Smooth muscle cell proliferation assay. TOP

Smooth muscle cells were isolated from rat aortas, cultured for 12-16 generations and maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% bovine calf serum and penicillin/streptomycin in a humidified, 5% CO₂ atmosphere at 37°C, as described previously (25). Cells were trypsinized, seeded onto 60-mm tissue culture dishes (Corning Glass Works, Corning, NY) at a density of 10 (5) cells per dish and cultured in minimum essential medium (MEM) supplemented with 10% serum and nonessential amino acids (100 µmol of

alanine, asparagine, aspartic acid, glutamic acid, proline, and serine) in the presence or absence of glycine (0.1–10 mmol/liter). On days 2, 4, 6, and 8, cells were trypsinized and counted.

Smooth muscle cell adhesion assay. TOP

Two six-well culture plates (60 mm) were precoated with 10 µg/ml of collagen (type 1, Collaborative Biomedical Products, Bedford, MA). Cells were resuspended in modified MEM (see above) with or without 10 mM glycine to give a final concentration of 2×10⁵ cells/ml. One ml of cells was added to each well and incubated in a humidified, 5% CO₂ atmosphere at 37°C. After 2.5 hr, nonadherent cells were removed, and the number of cells adhering to the bottom of the wells was determined microscopically (100×) by counting five randomly selected fields of constant area per well. All experiments were performed in triplicate.

Smooth muscle cell migration assay. TOP

Rat smooth muscle cells were preincubated in MEM supplemented with 10% serum and nonessential amino acids as described above in the presence or absence of glycine (10 mmol/liter) for 48 hr. The migration of smooth muscle cells was quantitated using a Transwell culture chamber (Costar, Cambridge, MA) where the upper and lower culture chambers are separated by a polycarbonate filter with 8-µm pores. Each membrane was coated with 30 µg/ml of rat tail collagen (Collaborative Biochemical Research) in 0.2 mol/liter acetic acid for 24 hr before each chemotactic assay. For each assay, 25 µl of PDGF (5 ng/ml, Life Technologies, Inc.) in MEM plus 10% serum was loaded in quadruplicate wells in the bottom chamber. Cultured smooth muscle cells with or without glycine (10 mmol/liter) were trypsinized and suspended at a concentration of 1×10⁵ cells/ml. A volume of 50 µl of the smooth muscle cell suspension was seeded in the upper chamber. The culture media with glycine (10 mmol/liter) was placed into the lower chamber of the well and cells preincubated with glycine were placed in the upper chamber. Control was glycine-free media and cells preincubated without glycine treated in the same manner. After 24 hr of incubation at 37°C, the filters were removed and the smooth muscle cells on the upper side of the filter were removed. The smooth muscle cells that had migrated to the lower side of the filter were fixed in methanol and stained with Diff-Quik staining solution (Dade International Inc., Miami, FL). The filters were excised from their plastic supports and mounted on glass slides with Permount (Sigma Chemical Co.). The number of migrating cells was determined microscopically by counting five high-power (400×) fields of constant area per well. The migration assays were performed with two wells per group each time and repeated a total of three times.

Statistics. TOP

All results were expressed as means±SEM. Statistical differences between means were determined using analysis of variance, analysis of variance on ranks or *t* test where appropriate. *P*<0.05 was selected before the study to determine statistical differences between groups.

RESULTS TOP

Glycine concentration in blood. TOP

Animals received either glycine-containing or control diets during the entire 10-week experimental period, and blood samples were taken at necropsy. The blood concentration of glycine from glycine-fed recipients was 0.56±0.06 mmol/liter, which was 3-fold higher than values of samples from rats fed control diet (0.18±0.03 mmol/liter, *P*<0.05).

The effect of dietary glycine on histological manifestations of transplant vasculopathy. TOP

Intimal proliferation, medial thinning, and perivascular inflammation are characteristics of allograft vasculopathy in this model (20, 23), and these pathological features of chronic rejection were found in our study. Figure 1 displays representative photomicrographs of aortic grafts stained with elastic-van Gieson. Isografts from rats fed either control or glycine diets were completely free of arteriosclerotic lesions, i.e., no intimal proliferation, medial necrosis, or adventitial inflammation was observed (Fig. 1A). In contrast, allografts from rats given control diet showed typical intimal thickening, medial thinning, and perivascular inflammation (Fig. 1B) and exhibited a pathology score of 3.7 (Table 1). The media showed loss of nuclei and breaks of elastic laminae, and in the neointima, nuclei accumulated in the subendothelial space. Intimal proliferation and medial necrosis were reduced dramatically by dietary glycine (Fig. 1C), with a nearly 50% lower pathology score (Table 1).

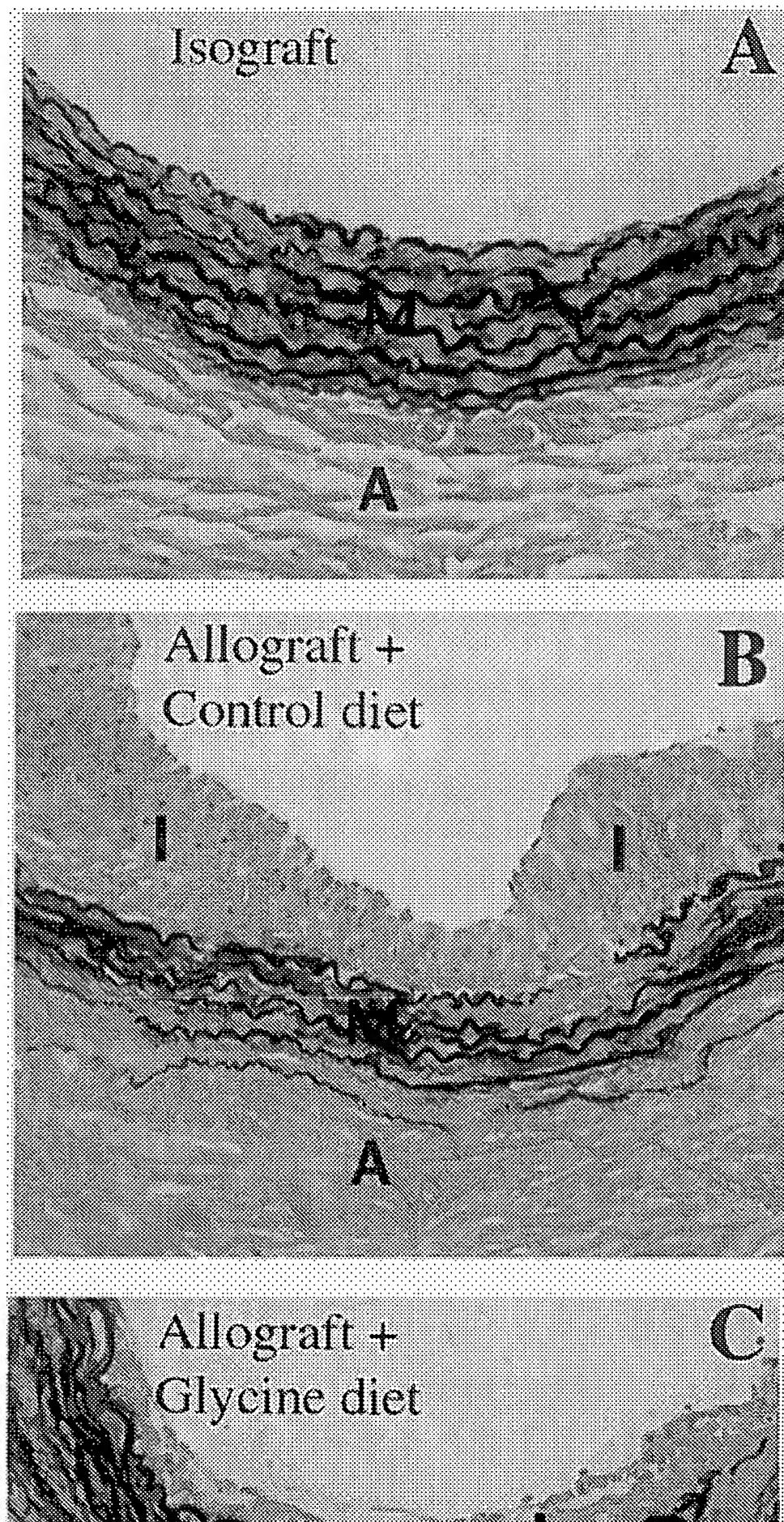


Figure 1. Representative photomicrographs of aortic grafts 10 weeks after transplantation. Lewis recipients of Lewis (syngenic transplantation) and Fisher-344 (allogenic transplantation) abdominal aortic grafts received either control or glycine-containing (5%) diets for 10 weeks. An isograft (A), an allograft from a rat fed control diet (B), and an allograft from a rat given glycine diet (C) are shown. Intima is marked I, media are labeled M and adventitia A. Animal treatments and surgical procedures are detailed in *Materials and Methods*. The grafts were stained with elastic-van Gieson. Original magnification, 100 \times .

TABLE 1. Effects of dietary glycine on cells in aortic grafts

Markers	Isografts ^a	Allografts + control diet	Allografts + glycine diet
Pathology score	0±0	3.7±0.2 ^b	2.0±0.3 ^{bc}
ED1 ^{+d}	0.5±0.3	43.5±4 ^b	27±4 ^{bc}
CD5 ^{+d}	0.3±0.3	135±20 ^b	138±17 ^b
PCNA ^{+e}	0.8±0.3	25±5 ^b	12±4 ^{bc}

Pathology was scored semiquantitatively as described in *Materials and Methods*.

^a There were no differences between control and glycine groups in isografts; therefore, values of these two groups were combined.

^b $P<0.05$ compared to isograft.

^c $P<0.05$ compared to allografts from rats fed control diet, by one-way analysis of variance using Tukey's post hoc test.

^d Monoclonal antibodies ED1 and CD5 recognize monocyte/macrophages and pan T lymphocytes, respectively.

^e PCNA is an abbreviation of proliferation cell nuclear antigen.

Table 1. Effects of dietary glycine on cells in aortic grafts Pathology was scored semiquantitatively as described in *Materials and Methods*.

^a There were no differences between control and glycine groups in isografts; therefore, values of these two groups were combined. ^b $P<0.05$ compared to isograft. ^c $P<0.05$ compared to allografts from rats fed control diet, by one-way analysis of variance using Tukey's post hoc test. ^d Monoclonal antibodies ED1 and CD5 recognize monocyte/macrophages and pan T lymphocytes, respectively. ^e PCNA is an abbreviation of proliferation cell nuclear antigen.

Image analysis was used to determine the area of each cell layer of the aortic grafts. Intimal areas (Fig. 2A) of isografts from both control and glycine-fed animals were nearly undetectable as expected ($0.01\pm0.01\times105\mu\text{m}^2$). However, the average intimal area of allografts from rats fed control diet for 10 weeks was increased to 2.1 ± 0.3 . Dietary glycine reduced the intimal area by 45% (1.2 ± 0.3 , $P<0.05$). In isografts, medial areas from rats given control diet were not different from those fed glycine-containing diet (2.9 ± 0.1 , Fig. 2B). The medial areas of allografts from animals given control diet were significantly smaller than the isografts (2.2 ± 0.2 , $P<0.05$). However, dietary glycine preserved the media (3.0 ± 0.1), yielding values that were comparable to the syngenic grafts and significantly higher than allografts from rats fed control diet. This led to intimal to medial ratios that were about two times larger in allografts from rats fed control diet than those fed glycine (0.9 ± 0.1 vs. 0.5 ± 0.1 , $P<0.05$) (Fig. 2C). Because the isograft intima consisted of a single layer of flat endothelial cells, the intima to media ratio was close to zero in syngenic transplants. These data demonstrate that glycine-containing diets can suppress the development of chronic rejection of aortic allografts.

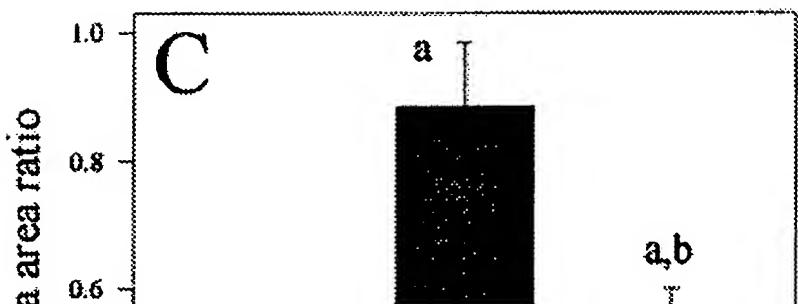
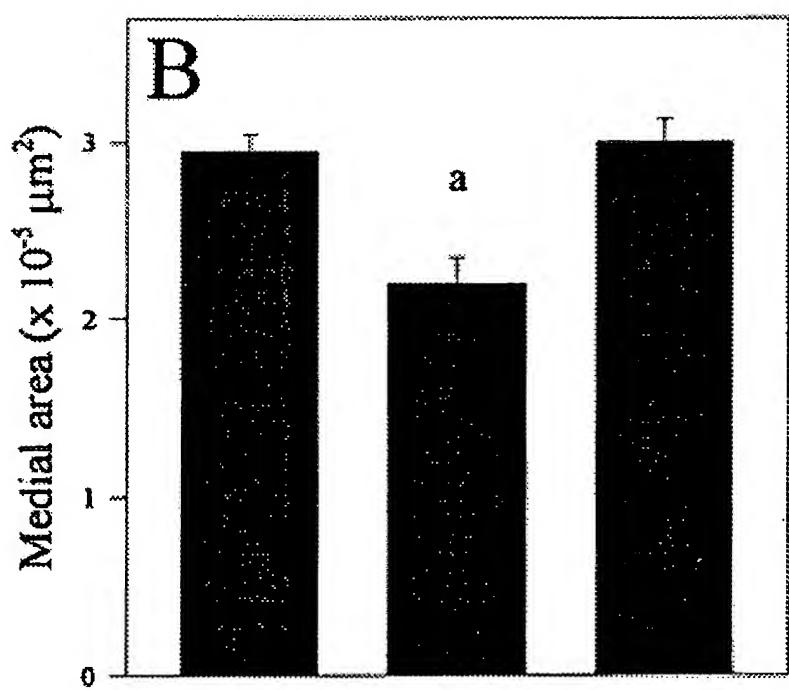
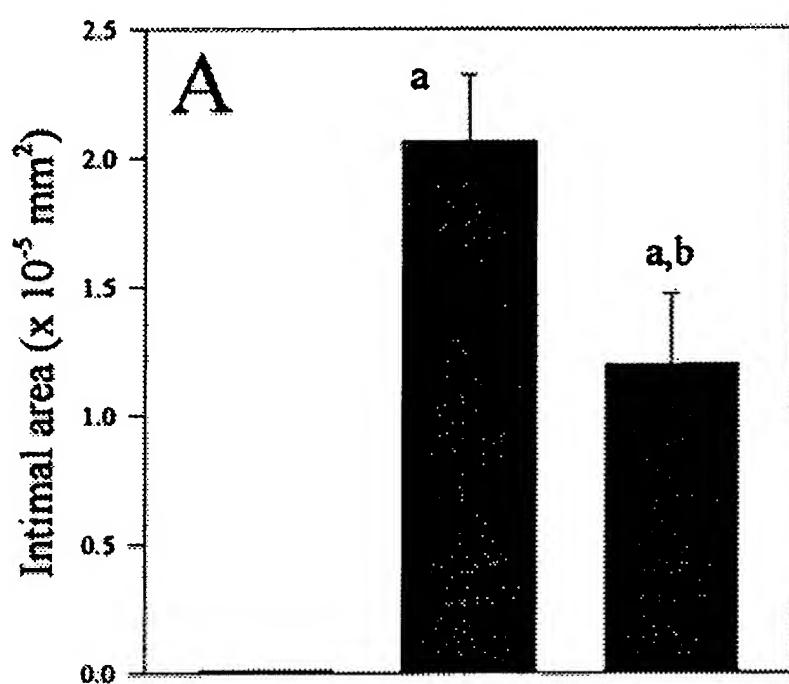


Figure 2. Effect of dietary glycine on vasculopathy of aortic grafts. Animal treatment and image analyses were performed as described in *Materials and Methods*. Intimal (A) and medial areas (B) were measured and the intima to media area ratio was calculated. Values represent mean \pm SEM. a, $P < 0.05$ for comparison with the isografts; b, $P < 0.05$ compared with the allografts from rats fed control diet by analysis of variance with Tukey's post hoc test.

The effect of dietary glycine on perivascular infiltration of aortic grafts. ^{TOP}

Figure 3 shows the magnitude of cellular infiltration in the adventitial layer. There were only a few scattered nuclei of fibroblasts in the perivascular areas of isografts fed control or glycine diets. As expected, a strong inflammatory response in allografts from rats fed control diet was observed, with a significant increase in nuclei in the adventitia. Dietary glycine blunted this increase in the number of perivascular leukocytes in the aortic allograft by about 40%.

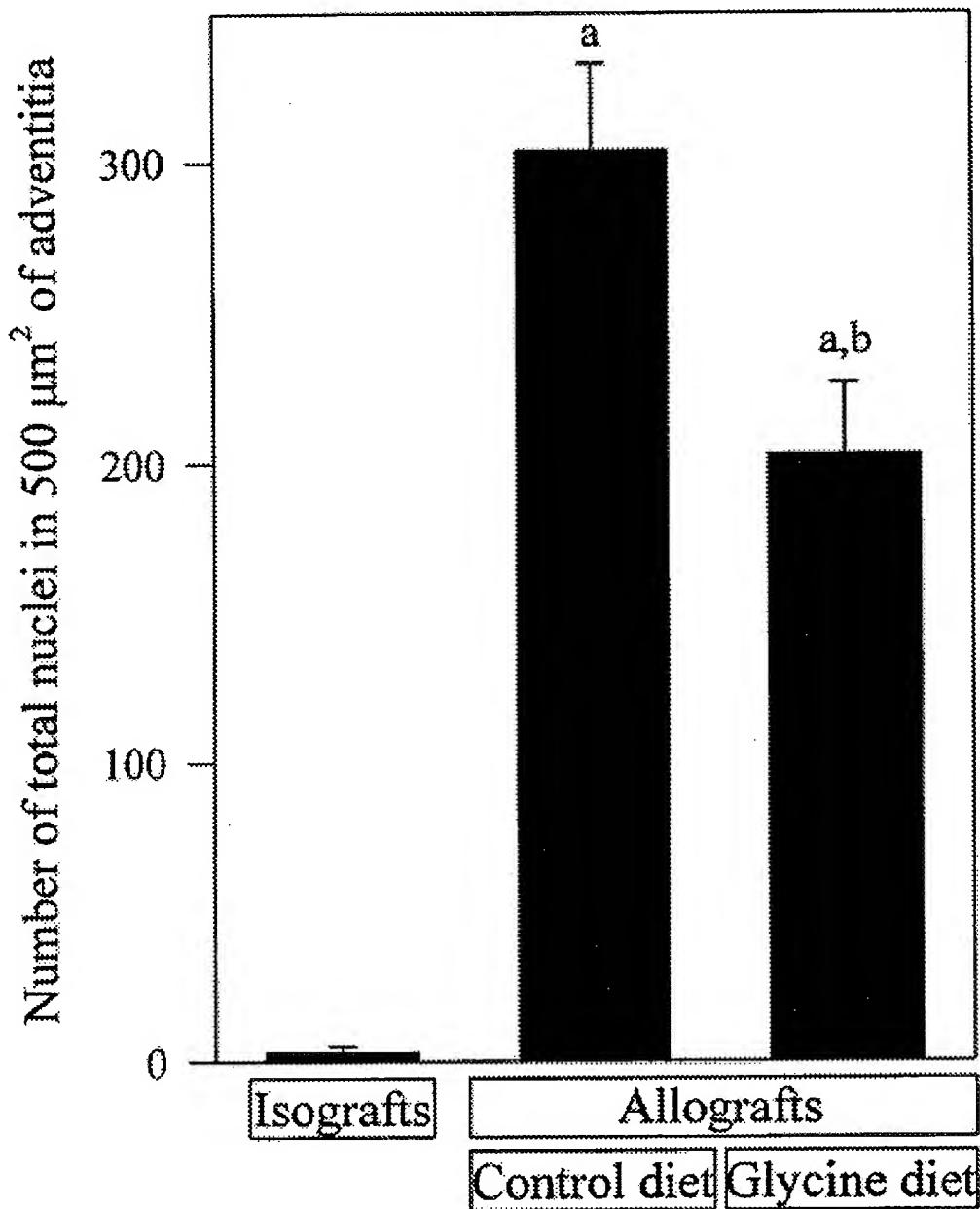


Figure 3. Effect of dietary glycine on perivascular inflammation of aortic grafts. Surgical procedures and animal treatments were described in *Materials and Methods*. Quantitation of infiltrating leukocytes, expressed by number of nuclei, was performed in hematoxylin and eosin stained grafts. Values represent mean \pm SEM. a, $P < 0.05$ compared with isografts; b, $P < 0.05$ compared to the allografts from rats fed control diet by analysis of variance with Tukey's post hoc test.

Specific immunostaining with antibodies against monocyte/macrophages ($ED1^+$) and T lymphocytes ($CD5^+$) was negative in isografts (**Table 1**). Allografts from rats fed control diet demonstrated significantly more perivascular infiltration of monocytes and macrophages than isografts. Dietary glycine, however, significantly reduced the perivascular accumulation of monocytes and macrophages (**Table 1**). CD5 staining showed significantly more T cell infiltration in allografts from rats given control diet than isografts, but this increase in T cells was not influenced by glycine.

Effects of dietary glycine on proliferation cell nuclear antigen in neointima. TOP

The ratio of $PCNA^+$ stained nuclei to total nuclei in neointimal areas of allografts with or without dietary glycine supplements was calculated and is shown in **Table 1**. There were almost no $PCNA^+$ nuclei in isografts. In contrast, about one-fourth of nuclei in allografts from rats fed control diet were $PCNA^+$, values which were about 2-fold higher than those in allografts from glycine-treated rats.

Effects of glycine on rat smooth muscle cell proliferation in vitro. TOP

To test the hypothesis that glycine minimizes aortic allograft vasculopathy by inhibiting smooth muscle cell proliferation, a series of in vitro experiments was performed. Indeed, when glycine (10 mmol/liter) was added to cultured smooth muscle cells, cell growth was inhibited in a time-dependent manner (**Fig. 4**). Significant differences between the control and glycine groups were observed as early as day 4, and on day 8, glycine suppressed growth of smooth muscle cells by ~50%. Next, an assay was performed to assess whether glycine affects the adherence of smooth muscle cells. No significant differences between the number of cells adhering to collagen-coated plates in the presence or absence of glycine (10 mmol/liter) were observed (data not shown). Moreover, culturing cells with glycine had no effect on cell morphology (**Fig. 5**). When the cells were incubated with different concentrations of glycine (0.1-10 mmol/liter) and cell number was determined from day 0 until day 8, a linear dose-dependent response was observed with an IC_{50} of about 1 mmol/liter (**Fig. 6**).

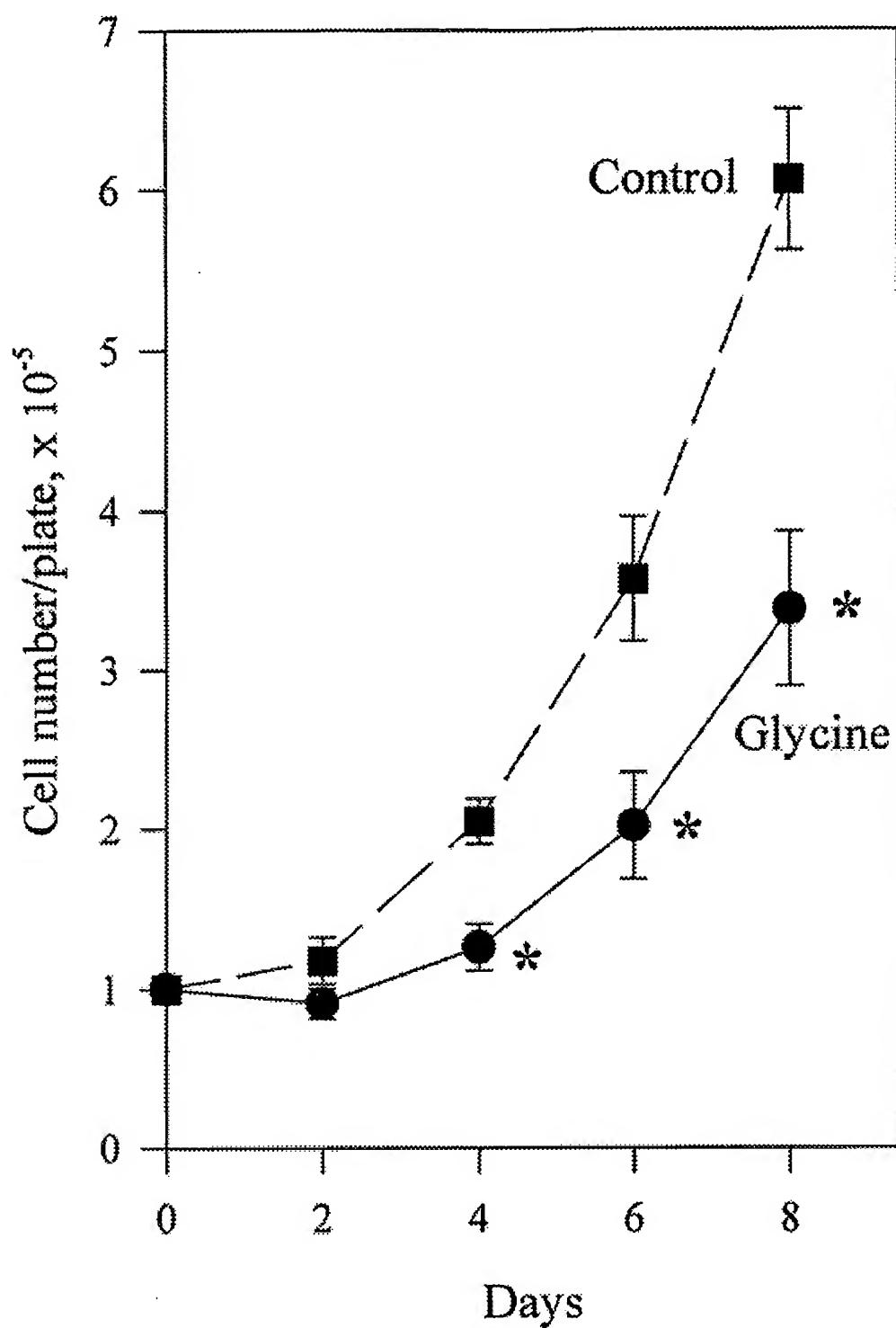
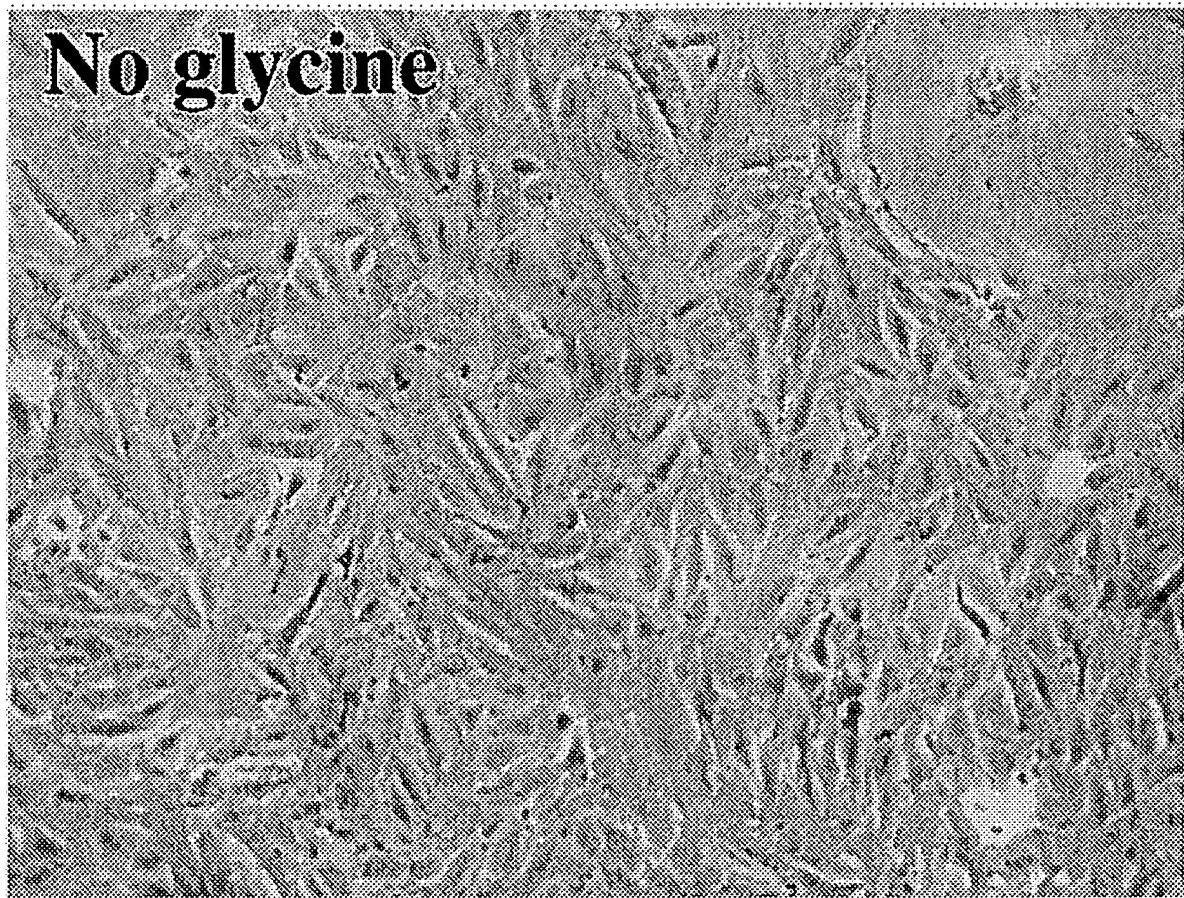


Figure 4. Inhibitory effects of glycine on growth of rat aorta smooth muscle cells in vitro. Rat aorta smooth muscle cells were plated at a density of 105 cells/dish in MEM supplemented with nonessential amino acids either with (○, solid line) or without (●, dotted line) glycine (10 mmol/liter) as described in *Materials and Methods*. Results are reported as mean \pm SEM from experiments performed in triplicate. Asterisks denote statistical differences from the corresponding time point ($P < 0.05$, by two-way analysis of variance).

No glycine



10 mmol/L glycine

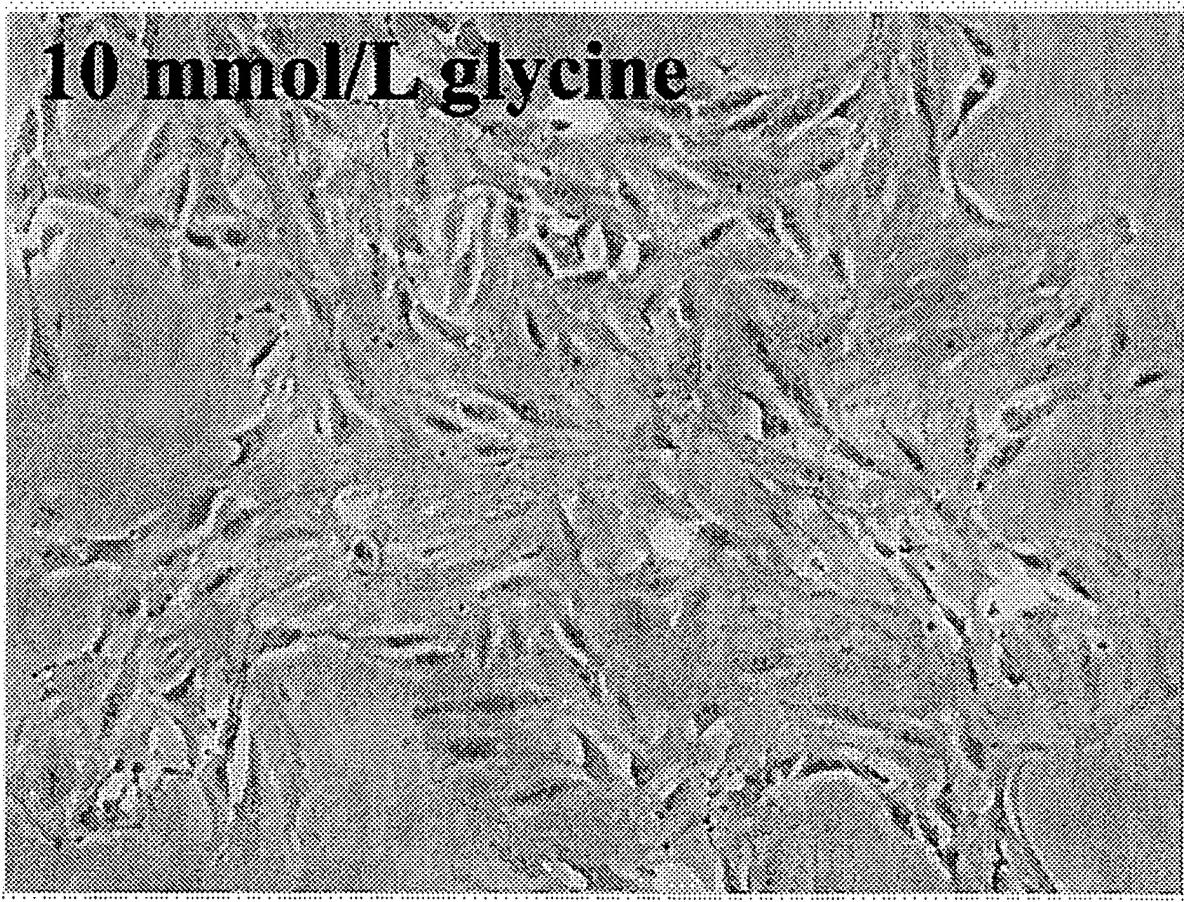


Figure 5. Effect of glycine on morphology of rat aortic smooth muscle cells in vitro. Representative photographs of smooth muscle cells in medium with or without glycine (10 mmol/liter). Rat aortic smooth muscle cells were seeded onto 60 mm/tissue culture dishes at a density of 1000 cells per dish and cultured with MEM in the presence or absence of glycine (10 mmol/liter) for 8 days. Cells were fixed and stained as detailed in *Materials and Methods*.

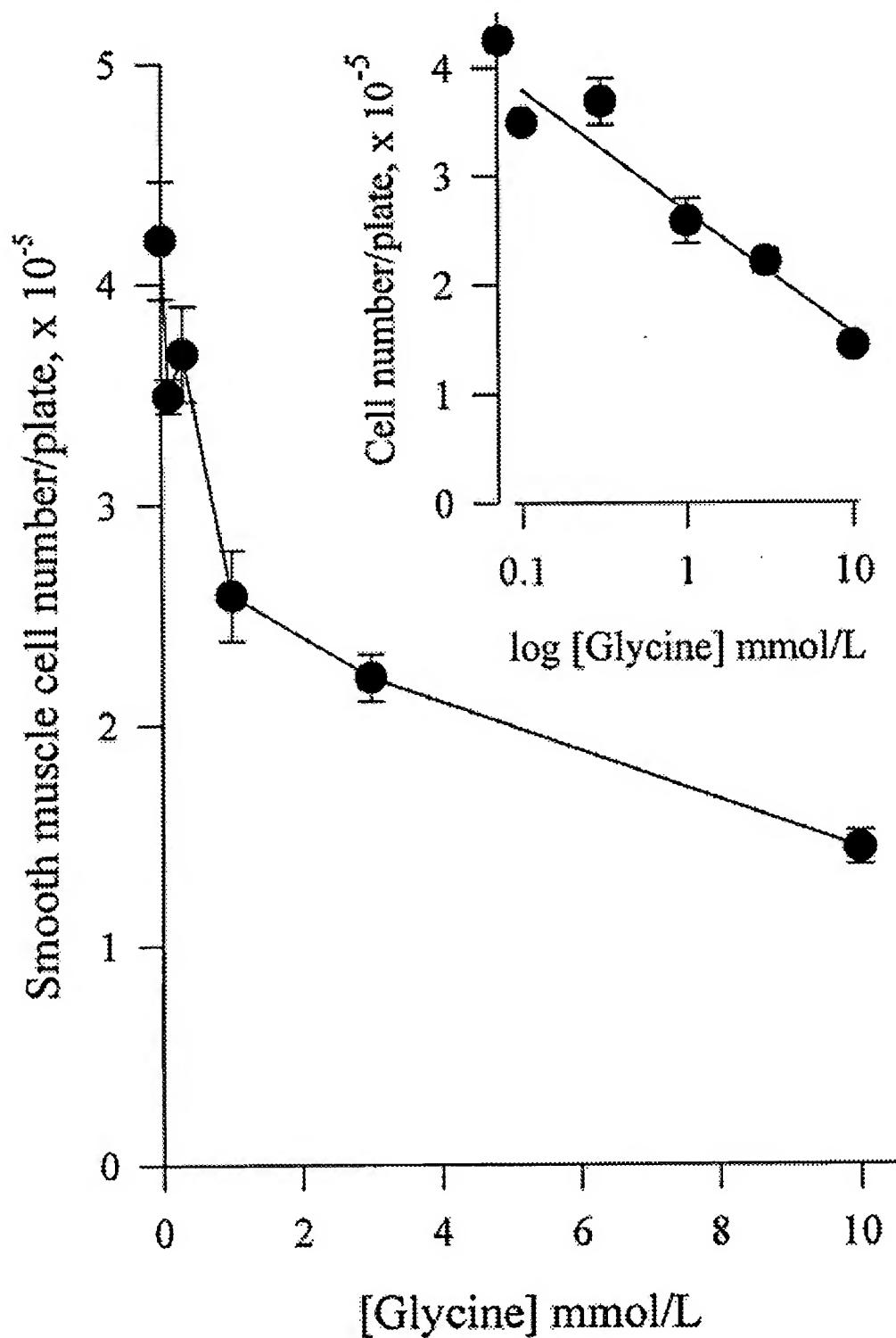


Figure 6. Inhibitory effect of glycine on in vitro growth of rat aorta smooth muscle cells is dose dependent. Rat aorta smooth muscle cells were plated at a density of 105 cells/dish in MEM supplemented with glycine (0.1–10 mmol/liter) as described in *Materials and Methods*. Cells were cultured for 8 days with one change of culture medium on day 4. At the end of the experiment, cells were detached by trypsinization and cell number determined. Results are reported as mean±SEM from experiments performed in triplicate. At all concentrations studied, differences from the control group were statistically significant (one-way analysis of variance with

Student-Newman-Keul's post hoc test).

Effects of glycine on rat smooth muscle cell migration in vitro.

As reported previously, cultured rat aortic smooth muscle cells migrate from the upper to the lower side of filters due to the chemotactic action of PDGF (26). Glycine (10 mmol/liter) added to culture media inhibited PDGF-induced migration of vascular smooth muscle cells by about 60% (Fig. 7), suggesting that glycine suppresses intimal thickening, at least partially, by inhibiting smooth muscle cell migration.

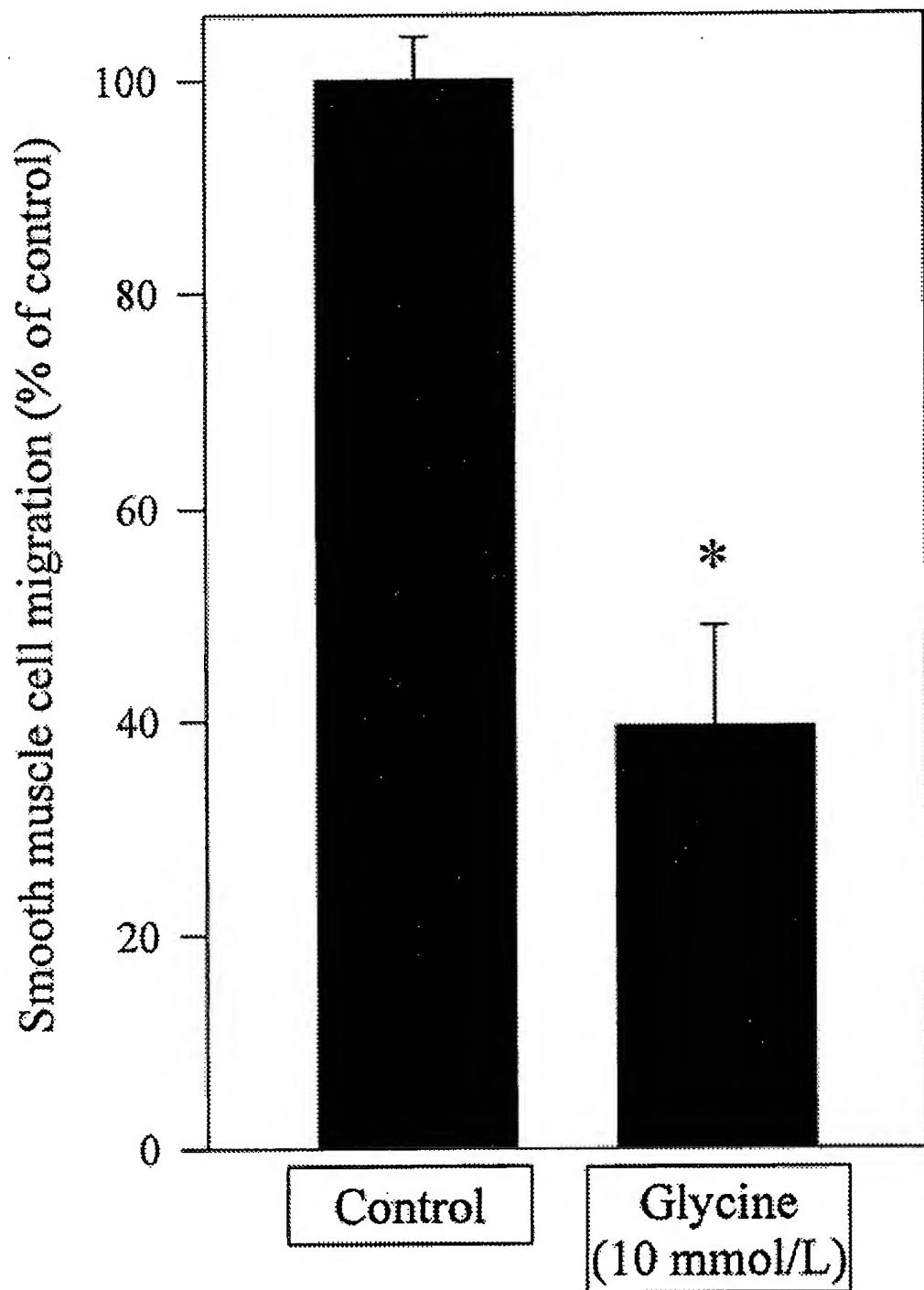


Figure 7. Inhibitory effects of glycine on migration of rat aorta smooth muscle cells in vitro. Rat aortic smooth muscle cell migration was determined as detailed in *Materials and Methods*. Cells were seeded in a Transwell culture chamber with a collagen-coated filter and grown in PDGF-containing culture medium with or without glycine (10 mmol/liter) for 24 hr. The smooth muscle cells that migrated to

the lower side of the filter were fixed, stained and counted as described in *Materials and Methods*. The asterisk indicates $P < 0.05$ compared with the number of smooth muscle cells that migrated in control medium by t test.

DISCUSSION TOP

Dietary glycine minimizes chronic rejection in aortic allografts. The essential features of human chronic rejection, or allograft vasculopathy, are persistent perivascular infiltrate associated with concentric intimal proliferation and medial necrosis (5). In our study using a model of Fisher-344 to Lewis aortic transplantation, similar lesions to human allograft vasculopathy were produced (Fig. 1). Intimal thickening, medial thinning and inflammation of the adventitia were observed in allografts, but not in isografts, confirming that antigen-dependent factors are critical in the pathogenesis of transplant vasculopathy. The mechanisms of intimal proliferation and medial thinning observed in allografts receiving control diet (compared to isografts) is unclear. Adventitia inflammation might be associated with necrosis of cellular components of the medial layer (23) or migration of smooth muscle cells from media into intima (27). Importantly, dietary glycine minimized transplant vasculopathy of aortic allografts. It reduced intimal proliferation, medial necrosis and perivascular inflammation significantly (Figs. 1–3, Table 1). Although allograft vasculopathy was not inhibited completely by dietary glycine, long-term dietary therapy is easy and exhibits no toxic side effects (28); therefore, it could be clinically useful. Moreover, dietary glycine minimizes CsA nephrotoxicity by reducing free radical production (16), which is an important risk factor in chronic rejection (24).

The mechanism of chronic rejection is poorly understood, but it most likely involves several cell types including endothelial cells and smooth muscle cells as well as leukocytes. Activation of the immune system is thought to be responsible for initiation and progression of chronic rejection and endothelial cells are the first target of the host immune system (29). Moreover, they undergo ischemia-reperfusion injury early after implantation, possibly enhancing antigenicity of the graft. This may cause up-regulation of MHC class II molecules and adhesion molecules leading to production of cytokines and growth factors, which facilitates infiltration of immune cells and migration and proliferation of smooth muscle cell (30, 31). Therefore, the protective effect of dietary glycine here might be due to effects on endothelial cells (32, 33). Moreover, humoral effects might be involved (34), and glycine inhibits B cell-mediated humoral reactions (35).

Cellular invasion in adventitia is one of the main features of chronic rejection and is regarded as a prerequisite for intimal thickening (23). When inflammatory cells infiltrate the blood vessels (36), they produce cytokines, growth factors, and chemotactic agents (37) that most likely are responsible for vascular smooth muscle cell proliferation and migration (3, 38). In addition, inflammatory leukocytes release cytokines and eicosanoids, leading to activation of graft vascular endothelium generating a vicious cycle, where increased expression of proinflammatory molecules leads to increased leukocyte extravasation, increased inflammation, and even increased expression of proinflammatory molecules (39). Subsequently, smooth muscle cells responding to these reactions and growth factors produced by endothelial cells, inflammatory cells, and smooth muscle cells themselves are induced to migrate into the media, replicate and begin remodelling the vascular wall. In the present study, dramatic perivascular inflammation was observed in aortic allografts (Fig. 3). Interestingly, dietary glycine decreased perivascular inflammation by reducing invasion of monocytes and macrophages, but had no effect on infiltration of T lymphocytes (Table 1). The early appearance of monocytes and macrophages in the allograft intima has been reported to be related to the development of chronic rejection (38, 40). The macrophage is able to process and present antigen, leading to the production of a variety of cytokines and growth factors, e.g., TNF- α and PDGF, that are mitogenic or cytotoxic (41, 42). Moreover, in the vascular wall of atherosclerosis, macrophages have been shown to produce matrix metalloproteinases (43) which are involved in the migration of smooth muscle cells in vascular lesions (44). It is possible, therefore, that glycine acts by inactivating monocytes and macrophages, leading to reduced smooth muscle cell migration from media to intima. This possibility is strengthened by the fact that glycine inactivates macrophages in the liver by activating a glycine-gated chloride channel (45). The lack of effectiveness of glycine on infiltration of T cells in this study may partially explain the fact that chronic rejection was not blocked completely in this model.

Glycine inhibited rat smooth muscle cell proliferation and migration. TOP

Vascular smooth muscle cell proliferation is a central event in the progression of chronic rejection (46). The increase in intimal thickness and cellularity was due, in large part, to proliferation of smooth muscle cells migrating from the media (47). The in vivo proliferative activity of smooth muscle cells in the neointima, as indicated by the number of PCNA⁺ cells in our study, was also reduced by glycine (Table 1). This is consistent with the hypothesis that glycine directly inhibits proliferation and migration of vascular smooth muscle cells of aortic allografts in addition to its effects on infiltrating macrophages. Indeed, glycine suppressed the growth of cultured vascular smooth muscle cells in a dose-dependent manner (Figs. 4 and 6) and inhibited the migration of cells in vitro (Fig. 7). It is known that changes in intracellular calcium are important for smooth muscle cells (48). Whether glycine influences the replication and migration of vascular smooth muscle cells by altering intracellular calcium through glycine-gated chloride channels, as is observed in resident hepatic macrophages (45), remains a possibility.

In conclusion, our results demonstrate that dietary glycine could be helpful as an adjuvant therapy to current immunosuppressive regimens to reduce development of chronic rejection. It is simple and lack of toxicity makes it possibly clinically useful.

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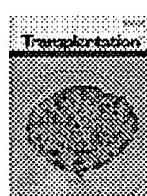
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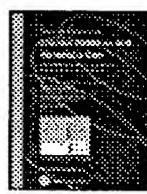
EFFECTS OF AGI-1096, A NOVEL ANTIOXIDANT COMPOUND WITH ANTI-INFLAMMATORY AND ANTIPIROLIFERATIVE PROPERTIES, ON RODENT ALLOGRAFT ARTERIOSCLEROSIS.

Transplantation. 77(10):1494-1500, May 27, 2004.

Murata, Seiichiro 1; Sundell, Cynthia L. 2; Lijkwan, Maarten A. 1; Balsam, Leora B. 1; Hammainen, Pekka 1; Coleman, Caroline 1; York, Chris 1; Luchoomun, Jayraz 2; Suen, Ki-Ling 2; Howard, Randy 2; Somers, Patricia K. 2; Morris, Randall E. 1; Robbins,

Robert C. 1,3

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L-Glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent.

Current Opinion in Clinical Nutrition & Metabolic Care. 6(2):229-240, March 2003.

Zhong, Zhi a; Wheeler, Micheal D. b; Li, Xiangli b; Froh, Matthias b; Schemmer, Peter b; Yin, Ming b; Bunzendauf, Hartwig c; Bradford, Blair d; Lemasters, John J. a

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Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice.

Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR.

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pennsylvania 15261, USA.

Understanding the immunological mechanisms of protection and pathogenesis in tuberculosis remains problematic. We have examined the extent to which tumor necrosis factor-alpha (TNF alpha) contributes to this disease using murine models in which the action of TNF alpha is inhibited. TNF alpha was neutralized in vivo by monoclonal antibody; in addition, a mouse strain with a disruption in the gene for the 55 kDa TNF receptor was used. The data from both models established that TNF alpha and the 55 kDa TNF receptor are essential for protection against tuberculosis in mice, and for reactive nitrogen production by macrophages early in infection. Granulomas were formed in equal numbers in control and experimental mice, but necrosis was observed only in mice deficient in TNF alpha or TNF receptor. TNF alpha and the 55 kDa TNF receptor are necessary conditions for protection against murine M. tuberculosis infection, but are not solely responsible for the tissue damage observed.

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